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Award Number: DAMD17-98-1-8319

TITLE: Cellular and Molecular Roles of the Akt Protein Kinase in Breast Carcinomas

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REPORT DATE: June 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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460 811

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 074-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503				
1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE June 2002	3. REPORT TYPE AND DATES COVERED Final (4 May 98 - 3 May 02)		
4. TITLE AND SUBTITLE Cellular and Molecular Roles of the Akt Protein Kinase in Breast Carcinomas		5. FUNDING NUMBERS DAMD17-98-1-8319		
6. Author(s) Anne B. Vojtek, Ph.D.				
7. Performing Organization Name (Include Name, City, State, Zip Code and Email for Principal Investigator) University of Michigan Ann Arbor, Michigan 48109-1274 E-Mail: avojtek@umich.edu		8. Performing Organization Report Number (Leave Blank)		
9. Sponsoring/Monitoring Agency Name and Address U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. Sponsoring/Monitoring Agency Report Number (Leave Blank)		
11. Supplementary Notes (i.e., report contains color photos, report contains appendix in non-print form, etc.)				
12a. Distribution/Availability Statement (check one) X Approved for public release; distribution unlimited			12b. Distribution Code (Leave Blank)	
13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) Akt is a serine/threonine kinase that has been implicated in the initiation and/or progression of breast cancers. In order to gain an understanding of how Akt promotes malignant transformation, we identified proteins that are regulated by Akt, including the Brn-1 transcription factor, the B-Raf serine/threonine kinase, and the Elk-1 transcription factor. Brn1 co-associates with Akt1 in vivo and is phosphorylated in vitro by Akt1. The site of phosphorylation of Brn1 by Akt1 was mapped to S407. This site is conserved in POU domain family members, suggesting a general role for Akt in their biology. Akt negatively regulates the enzymatic activity of B-Raf in vitro and in vivo by phosphorylating two residues in the amino-terminal regulatory domain of B-Raf. Akt also negatively regulates the activity and level of the Elk-1 transcription factor. Thus, Akt may promote transformation and cell survival in part by altering gene expression through regulation of transcription factors and in part by regulating the Ras/Raf pathway by phosphorylating and downregulating the activity of the B-Raf kinase.				
20021118 047				
14. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award) Breast Cancer, Akt kinase, Raf kinase			15. Number of Pages (count all pages including appendices) 23	
			16. Price Code	
17. Security Classification of Report Unclassified	18. Security Classification of this Page Unclassified	19. Security Classification of Abstract Unclassified	20. Limitation of Abstract Unlimited	

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Introduction

The Akt serine/threonine kinase was first identified as a viral transforming agent of a T-cell lymphoma in an AKR mouse (AKR mouse T cell lymphoma, or Akt) (1). Subsequent studies revealed a critical role for Akt in the initiation and/or progression of breast carcinomas (2, 3). The cellular and molecular events that are altered upon the constitutive activation of Akt are not completely understood. Akt could initiate and/or promote the progression of breast cancer by phosphorylating key intermediates in growth signaling pathways. Alternatively, Akt could promote cell survival by phosphorylating and inactivating key components of an apoptosis pathway. To increase our understanding of the role that Akt plays in breast carcinomas, we used molecular, cellular and biochemical approaches to identify and characterize regulators and effectors of Akt.

Note

No dollars for supplies or manpower were expended since 9/30/01.

Work Accomplished to Date

Our work over the past several years has resulted in the identification of new effector (target) proteins for Akt1 (Task1). The new effectors that we identified include the Brn-1 transcription factor, the B-Raf serine/threonine kinase, and the Elk-1 transcription factor. Our work on each of these effectors is summarized below and in Figure 1.

We identified a transcription factor, the Brn1 protein, as a likely downstream target of Akt1 (June 1999 annual report). We demonstrated that Brn1 co-associates with Akt1 in vivo and is phosphorylated in vitro by Akt1. The site of phosphorylation of Brn1 by Akt1 was mapped to S407. This site is conserved in POU domain family members, suggesting a general role for Akt in their biology. Pou domain transcription factors play an important role in cell differentiation and cell survival (4). In addition, one family member Brn-3a cooperates with the Ras oncogene to transform mammalian cells (5). Thus, Akt may promote transformation and induce cell survival by regulating the transcriptional program induced by POU domain family members.

We also identified the B-Raf serine/threonine kinase as a downstream target of Akt (June 2000 annual report). B-Raf contains multiple Akt phosphorylation consensus sites in its amino terminal regulatory domain. Mutational analysis demonstrated that Akt phosphorylates B-Raf at residues S364 and S428. The alteration of these phosphoacceptor sites from serine or threonine to alanine resulted in a progressive increase in B-Raf enzymatic activity in vitro and in vivo. Furthermore, we demonstrated that ectopic expression of constitutively active Akt inhibits the ability of epidermal growth factor to activate B-Raf enzymatic activity and that inhibition of the endogenous Akt signaling pathway with LY294002 upregulates B-Raf enzymatic activity. Taken together, these results suggest that Akt negatively regulates B-Raf in vivo. Our research on the regulation of B-Raf by Akt was published in the *Journal of Biological Chemistry* [6, see appendix].

The Elk protein is a downstream target of the Ras oncogene and is implicated in the activation of the c-fos immediate early gene (7, 8). We have found that Akt negatively regulates the activity of the Elk-1 transcription factor. Our working model is that Akt uncouples Elk-1 from the transcriptional co-activators CBP/p300 and/or Sur2. In addition, we have found that in the presence of a constitutively active Akt, the levels of the Elk-1 protein are dramatically decreased. We have mapped this effect of Akt to the Elk-1 DNA binding domain, amino acids 1

to 93. This domain is conserved among the ternary complex transcription factors, Elk-1, Sap1a and Sap2/Erp/Net. As predicted, the levels of the Sap1a transcription factor are substantially lower in the presence of activated Akt, suggesting conservation of this action of Akt on multiple members of this family of transcription factors. In the MCF7 breast cancer cell line, ectopic expression of Elk promotes cell death in response to calcium ionophore treatment (9). In addition, c-fos, a transcriptional target of Elk-1, is associated with cell death in a number of different systems (10, 11). Thus, Akt may promote cell survival in part by inactivating Elk-1 transcriptional activity and decreasing the level of the Elk-1 protein. A manuscript describing our research on the regulation of Elk-1 by Akt is in preparation.

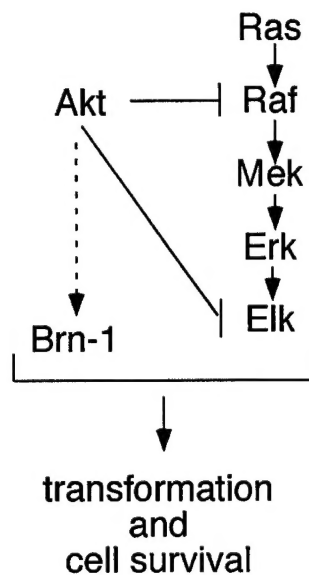


Figure 1. Akt effectors. To begin to elucidate the mechanism by which Akt promotes breast cancer, we identified effectors for Akt. Akt co-associates with and phosphorylates the Brn-1 transcription factor. Akt phosphorylates and negatively regulates B-Raf enzymatic activity in vitro and in vivo. Akt negatively regulates the activity of the Elk-1 transcription factor and also regulates the level of the Elk-1 protein in vivo. By regulating multiple effector pathways (transcriptional events and the kinase cascade downstream of the Ras oncogene), Akt promotes transformation and cell survival.

We also proposed to identify and characterize regulators of Akt (Task 1). Akt is activated by the lipids synthesized by phosphatidylinositol 3-kinase (PI3K), phosphatidylinositol 3,4 biphosphate and phosphatidylinositol 3,4,5 trisphosphate. The Ras and Rho family of small GTPases are regulators of the enzymatic activity of PI3K. Thus, events that regulate the activity and/or subcellular location of Ras and Rho proteins are likely to play a critical role in regulating the PI3K/Akt pathway. We have demonstrated that prenylated Rab acceptor protein 1 (PRA1) binds farnesylated and geranylgeranylated small GTPases, including Ras and Rho. We propose that PRA1 acts as an escort protein for small GTPases by binding to and "solubilizing" the hydrophobic isoprenoid moieties of the small GTPases and that this association facilitates trafficking of the small GTPases through the endomembrane system. Perhaps inhibitors that block the interaction of PRA1 with small GTPases may inhibit tumorigenesis and metastasis involving the Ras/Rho/PI3K/Akt pathway. This study on the association of PRA1 with Ras and Rho was published in the *Journal of Biological Chemistry* [ref 12, see appendix].

From May 1998 through September 2001 (the funding period of DAMD17-98-1-8319), we completed Task 1, identification of regulators and effectors of Akt. We performed a yeast two-hybrid screen (Task 1, part a), sorted the library positives into classes (Task 1, part b), restriction mapped and sequenced library positives (Task 1, part c), characterized the interaction of Akt and

its partners in vitro (Task 1, part d), performed in vitro kinase assays (Task 1, part e), and obtained full length clones of interacting proteins (Task 1, part f). We also demonstrated that Akt uses multiple effector pathways, Task 2. We constructed plasmids for expressing the Akt interacting proteins in tissue culture cells (Task 2, part a) and examined changes in gene expression (Task 2, part b). We published the following two manuscripts (Task 2, part d):

1. Guan, K-L., Figueroa, C., Brtva, T., Taylor, J., Barber, T., Zhu, T. and Vojtek, A. (2000). Negative regulation of the B-Raf serine/threonine kinase by Akt. *J. Biol. Chem.* **275**, 27354-59.
2. Figueroa, C., Taylor, J., and Vojtek, A. B. (2001). PRA1 is a receptor for prenylated small GTPases. *J. Biol. Chem.* **276**, 28219-28225.

We expect to submit a manuscript on the role of Akt1 in regulating Elk-1 by the end of June 2002 (Figueroa, Taylor and Vojtek, "Akt regulates the Elk-1 transcription factor," in preparation).

Key Research Accomplishments

- Identified the Brn-1 transcription factor as an Akt interacting protein, using a yeast two-hybrid screen
 - Provided secondary evidence of complex formation between Akt and Brn-1
 - Demonstrated that S407 in Brn-1 is phosphorylated by Akt
 - Taken together, these results suggest that the activity of the Brn-1 transcription factor is likely to be regulated by Akt
-
- Demonstrated that Akt and B-Raf co-associate in vivo
 - Demonstrated that B-Raf is phosphorylated by Akt at multiple residues within its amino terminal domain (S364 and S428)
 - Demonstrated that alteration of the serine residues within the Akt consensus sites in B-Raf to alanine results in a progressive increase in enzymatic activity in vitro and in vivo
 - Expression of Akt inhibits EGF induced B-Raf activity and inhibition of Akt with LY294002 upregulates B-Raf activity, suggesting that Akt negatively regulates B-Raf in vivo
 - Taken together, these results show that the B-Raf kinase is negatively regulated by Akt in vivo and in vitro
-
- Demonstrated that Akt expression blocks induction of Elk-1-dependent transcription activity by activated Mek
 - Demonstrated that Akt does not block extracellular regulated kinase (Erk) activation nor does Akt reduce Erk activity
 - Demonstrated that Akt reduces the level of Elk but not an internal control, kinase suppressor of Ras (KSR).
 - Demonstrated that activated Akt also reduces the level of the Sap1a protein
 - Identified amino acids 1 to 93 of Elk-1 as the domain that mediates the effects of Akt on Elk-1 protein levels
 - Taken together, these results suggest that the Elk-1 transcription factor is negatively regulated by Akt

Reportable Outcomes

1. Bibliography of Publications

Guan, K-L., Figueroa, C., Brtva, T., Taylor, J., Barber, T., Zhu, T. and Vojtek, A. (2000). Negative regulation of the B-Raf serine/threonine kinase by Akt. *J. Biol. Chem.* **275**, 27354-59.

Figueroa, C., Taylor, J., and Vojtek, A. B. (2001). PRA1 is a receptor for prenylated small GTPases. *J. Biol. Chem.* **276**, 28219-28225.

2. Funding Applied For Based on Work Supported by this Award
American Cancer Society Research Scholar Grant
7/01-6/04

3. Degrees Obtained

Claudia Figueroa is expected to receive her Ph.D. in October 2002

Due to family obligations (birth of her second child), Ms. Figueroa's dissertation defense was delayed to October 2002.

4. List of Meeting Abstracts

Era of Hope Department of Defense Breast Cancer Research Program
Meeting, June 8-11, 2000

5. List of Personnel Receiving Pay from DAMD17-1-98-8319

Anne B. Vojtek, Assistant Professor

Claudia Figueroa, Graduate Student

Jennifer Taylor, Research Associate

May Tsoi, Lab Aid/Research Assistant

Tamara Kouskoulas, Undergraduate Work Study Student

Conclusions

The Akt serine/threonine kinase plays a critical role in initiation and/or progression of breast carcinomas. To elucidate the mechanism by which Akt promotes breast cancer, we identified effectors for Akt. First, we found that Akt phosphorylates and likely regulates the transcriptional activity of POU domain transcription factors. Second, we demonstrated that Akt phosphorylates and negatively regulates B-Raf enzymatic activity in vitro and in vivo. Third, we observed that Akt negatively regulates the activity of the Elk-1 transcription factor and that Akt regulates the level of the Elk-1 protein in vivo. Thus, Akt regulates multiple effector pathways, both transcriptional events and the kinase cascade downstream of the Ras oncogene. The coordinate regulation of these multiple effector pathways by Akt is likely to play a critical role in cancer initiation and progression.

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12. C. Figuerao *et al.*, *J. Biol. Chem.* **276**, 28219-28225 (2001).

Appendix

Two publications:

1. Guan, K-L., Figueroa, C., Brtva, T., Taylor, J., Barber, T., Zhu, T. and Vojtek, A. (2000). Negative regulation of the B-Raf serine/threonine kinase by Akt. *J. Biol. Chem.* **275**, 27354-59.
2. Figueroa, C., Taylor, J., and Vojtek, A. B. (2001). PRA1 is a receptor for prenylated small GTPases. *J. Biol. Chem.* **276**, 28219-28225.

Negative Regulation of the Serine/Threonine Kinase B-Raf by Akt*

Received for publication, May 22, 2000, and in revised form, June 23, 2000
Published, JBC Papers in Press, June 26, 2000, DOI 10.1074/jbc.M004371200

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B-Raf contains multiple Akt consensus sites located within its amino-terminal regulatory domain. One site, Ser³⁶⁴, is conserved with c-Raf but two additional sites, Ser⁴²⁸ and Thr⁴³⁹, are unique to B-Raf. We have investigated the role of both the conserved and unique phosphorylation sites in the regulation of B-Raf activity *in vitro* and *in vivo*. We show that phosphorylation of B-Raf by Akt occurs at multiple residues within its amino-terminal regulatory domain, at both the conserved and unique phosphorylation sites. The alteration of the serine residues within the Akt consensus sites to alanines results in a progressive increase in enzymatic activity *in vitro* and *in vivo*. Furthermore, expression of Akt inhibits epidermal growth factor-induced B-Raf activity and inhibition of Akt with LY294002 up-regulates B-Raf activity, suggesting that Akt negatively regulates B-Raf *in vivo*. Our results demonstrate that B-Raf activity can be negatively regulated by Akt through phosphorylation in the amino-terminal regulatory domain of B-Raf. This cross-talk between the B-Raf and Akt serine/threonine kinases is likely to play an important role in modulating the signaling specificity of the Ras/Raf pathway and in promoting biological outcome.

Diverse extracellular stimuli, including growth factors, cytokines, and hormones, promote the formation of active, GTP-Ras. GTP-Ras directly interacts with the Raf family of serine threonine kinases and type I phosphatidylinositol 3-kinases (PI3K)¹ (1–3). Upon activation, Raf phosphorylates mitogen-activated protein kinase/extracellular signal-regulated kinase, which in turn phosphorylates and activates ERK1/2. The ERKs phosphorylate cytoplasmic targets, including the kinases Rsk and Mnk, and translocate to the nucleus where they stimulate the activity of various transcription factors, such as Elk-1, Fos, Jun, and Myc (4). Activation of phosphatidylinositol 3-kinase by both Ras-dependent and Ras-independent mechanisms leads to the increased production of phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-

trisphosphate. These lipids regulate the activity and/or localization of a number of target proteins, including those that contain pleckstrin homology domains. One such pleckstrin homology domain-containing protein regulated by lipids is the serine/threonine kinase Akt (also called protein kinase B). Akt was identified as the viral transforming agent of a T-cell lymphoma, and subsequent studies revealed a central role for Akt in promoting cell survival (5). Targets for Akt include kinases, glycogen-synthase kinase 3 and p70S6 kinase, transcription factors, FKHR and cAMP-response element-binding protein, as well as proteins associated with apoptosis, caspase 9 and BAD (6).

Ras promotes cell growth, in part by activation of the Raf/ERK pathway, and unrestrained activation of the Ras pathway is a common occurrence in many human tumors (7). In addition to its role in cell growth, Ras promotes cell survival through activation of the Raf/ERK and PI3K/Akt cascades (8). Ras also regulates differentiation; in the committed neuronal PC12 cell line, Ras/Raf promotes differentiation, whereas in C2C12 myoblasts, the Ras/Raf/ERK pathway blocks skeletal muscle differentiation (9, 10). Ras also promotes cell senescence and cell death, through activation of the Raf kinases (8). Thus, many contrary effects of Ras, promoting differentiation *versus* blocking differentiation and promoting cell proliferation, cell death, or cell survival, require the action of the Raf/ERK pathway. Cross-talk between signaling pathways is likely to be one mechanism by which such divergent biological outcomes are achieved through the use of the Ras signaling pathway. The presence of three Akt consensus sites in the amino-terminal regulatory domain of B-Raf led us to investigate cross-talk between the Ras/Raf pathway and Akt.

Mammalian cells contain three Raf proteins: c-Raf (or Raf-1), A-Raf, and B-Raf. B-Raf exists in multiple spliced forms, which exhibit tissue-specific expression patterns (11). Although all three Rafs are activated by receptor tyrosine kinases through their ability to associate with Ras, the three isoforms display differences in their regulation. Maximal activation of B-Raf requires only signals that activate Ras, whereas maximal activation of c-Raf and A-Raf require signals that activate Ras and signals that lead to their phosphorylation at tyrosine residue 341 (12, 13). Moreover, in PC12 cells, the sustained activation of ERKs in response to nerve growth factor is mediated by Rap1 acting not on c-Raf but on B-Raf (9). Thus, the three Raf proteins are differentially regulated.

Much is known about the role of phosphorylation in c-Raf regulation. Both stimulatory and inhibitory sites have been identified, and sites for serine/threonine and tyrosine phosphorylations have been mapped in c-Raf. The regulation of B-Raf by phosphorylation has diverged considerably from that of c-Raf. c-Raf contains tyrosine residues at 340 and 341, and Tyr³⁴¹ is the major site of tyrosine phosphorylation when c-Raf is coexpressed with activated Ras and Src in mammalian cells

* This work was supported by grants from the American Diabetes Association (to A. B. V.), the United States Army Medical Research and Materiel Command Grant DAMD17-98-1-8319 (to A. B. V.), a National Research Service Award postdoctoral fellowship (to T. R. B.), and Public Health Service Grant GM51586 (to K.-L. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; HEK293 cells, human embryonic kidney 293 cells; HA, hemagglutinin; MOPS, 4-morpholinepropanesulfonic acid; K/D, kinase dead.

(13). In contrast, B-Raf does not have tyrosines at the positions equivalent to 340 and 341, but rather aspartic acid occupies these positions (Asp⁴⁴⁷ and Asp⁴⁴⁸). These aspartic acid residues in B-Raf contribute to the elevated basal kinase activity observed with B-Raf (13). The elevated kinase activity of B-Raf is normally 15–20 times higher than an equivalent amount of c-Raf. Thus, in striking contrast to c-Raf, tyrosine kinases are not involved in the activation of B-Raf. Moreover, Ser⁴⁴⁵ in B-Raf (the equivalent residue in c-Raf is Ser³³⁸) is constitutively phosphorylated (13). In c-Raf, there is a low level of phosphorylation at Ser³³⁸ in serum-starved cells, which is then greatly increased following stimulation with growth factors, including epidermal growth factor (14). Alteration of Ser⁴⁴⁵ in B-Raf reduces its basal kinase activity; thus, constitutive phosphorylation of B-Raf at Ser⁴⁴⁵, together with the aspartic acid residues at 447 and 448, is responsible for the elevated basal kinase activity of B-Raf.

Recently, while the work that is presented here was in progress, Rommel *et al.* (15) demonstrated that in one biological cell context, that of muscle cell hypertrophy, the Ras/Raf/ERK pathway and Akt have opposing effects. In addition, Zimmerman and Moelling (16) showed that Akt could phosphorylate c-Raf at Ser²⁵⁹. The alteration of Ser²⁵⁹ to A resulted in a relatively modest 2-fold increase in enzymatic activity. Phosphorylation of Ser²⁵⁹ in the amino-terminal domain of c-Raf has been previously shown to decrease the enzymatic activity of c-Raf by promoting its association with 14-3-3 proteins (12). The serine residue at 259 in c-Raf is conserved in B-Raf, Ser³⁶⁴, and as in c-Raf, this residue is part of a consensus site for Akt phosphorylation. Intriguingly, B-Raf has two additional Akt consensus sites, Ser⁴²⁸ and Thr⁴³⁹. Moreover, the two Akt consensus sites unique to B-Raf do not meet the consensus for 14-3-3 binding.

Here we demonstrate that B-Raf is phosphorylated by Akt. In contrast to c-Raf, phosphorylation of B-Raf occurs at multiple residues within its amino-terminal regulatory domain. The alteration of the serine residues within the Akt consensus sites to alanines results in a progressive increase in enzymatic activity both *in vitro* and *in vivo*. Our results demonstrate that B-Raf activity can be negatively regulated by Akt through phosphorylation in the amino-terminal regulatory domain of B-Raf.

EXPERIMENTAL PROCEDURES

Plasmids and Mutagenesis—B-Raf was expressed as a fusion protein in HEK293 cells to either glutathione *S*-transferase (GST) in the expression vector pEBG-4X or to HA in the expression vector pcDNA3 (Invitrogen). Mutations in B-Raf at the ATP binding site (K482M) and at the Akt consensus phosphorylation sites were generated by site-directed mutagenesis and confirmed by restriction enzyme and sequence analysis.

Kinase Assays—For HA-B-Raf activity assays, 100 ng of pcDNA3-HA-B-Raf was transfected into HEK293 cells in 6-well plates using LipofectAMINE (Life Technologies, Inc.). 24 h after transfection, cells were starved in 0.1% fetal bovine serum medium for 12 h. Cells were lysed in radioimmune precipitation buffer. HA-B-Raf was immunoprecipitated with 2 μ g of anti-HA antibody (BabCo). The immunoprecipitated HA-B-Raf was assayed by a coupled *in vitro* kinase assay (17). Briefly, 0.1 μ g of GST-mitogen-activated protein kinase/extracellular signal-regulated kinase 1 was incubated with the precipitated HA-B-Raf in 20 μ l of kinase buffer for 20 min at 30 °C. The reaction was briefly centrifuged to pellet the HA-B-Raf, which was bound to protein G-agarose. 15 μ l of the supernatant was mixed with 0.1 μ g of GST-ERK1 in 20 μ l of kinase reaction buffer and incubated for 10 min at 30 °C. Then 10 μ l of a mixture containing 3 μ g of GST-Elk-1 and [γ -³²P]ATP was added to the reaction and incubated for 20 min at 30 °C. Phosphorylation of GST-Elk was determined by SDS-polyacrylamide gel electrophoresis and phosphorimager analysis.

Myc-ERK plasmid (300 ng) was transfected into HEK293 cells in the presence or absence of B-Raf or Ras. 24 h after transfection, cells were starved in 0.1% fetal bovine serum medium for 12 h. Myc-ERK were

immunoprecipitated, and kinase activity was determined using GST-Elk as a substrate (18). The amount of Myc-ERK used for kinase assays was analyzed by anti-ERK immunoblot.

For the Akt kinase assays, HEK293 cells in 60 mm dishes were transfected with 6 μ g of pCS2+-N-FLAG-Akt using a calcium phosphate transfection method. 48 h after transfection, cells were starved for 16 h in 0.1% fetal bovine serum medium. Following treatment with 100 mM LY294002 (or Me₂SO vehicle) for 1 h, cells were stimulated with insulin (20 μ g/ml medium; 3.5 μ M) for 10 min and immediately lysed in buffer A (19). Clarified extracts were incubated for 2 h with anti-FLAG M2-agarose resin to purify FLAG-Akt. The immunoprecipitated Akt was washed three times in buffer A containing 0.5 M NaCl, twice with buffer B, and once with assay dilution buffer before being evenly aliquotted to tubes for kinase assay. GST-tagged, kinase-inactive B-Raf and B-Raf mutant cDNAs (15 μ g) were transfected into 100-mm dishes of HEK293 cells. 36 h after transfection, actively growing cells were lysed in radioimmune precipitation buffer, and GST-B-Raf proteins were collected with glutathione-Sepharose beads. The beads were washed three times in buffer A containing 0.5 M NaCl, twice with buffer B, and GST-B-Raf substrates were then eluted using 10 mM glutathione in 50 mM Tris (pH 8.0) and quantitated using a Bradford assay (Bio-Rad). Approximately 1.0 μ g of each GST-B-Raf mutant was incubated with immunoprecipitated Akt at 30 °C for 30 min in a kinase reaction containing 6.7 mM MOPS (pH 7.2), 8.3 mM β -glycerol phosphate (pH 7.0), 0.33 mM Na₃VO₄, 0.33 mM dithiothreitol, 25 mM MgCl₂, 167 mM ATP, and 10 μ Ci of [γ -³²P]ATP. One-third of the reaction product was subjected to SDS-polyacrylamide gel electrophoresis followed by transfer to Immobilon filter, phosphorimager analysis, and Western blotting to verify equivalent GST-B-Raf substrate levels.

Coimmunoprecipitation of B-Raf and Akt—GST-Akt and HA-B-Raf were cotransfected into HEK293 cells using a calcium phosphate transfection protocol. Approximately 36 h after transfection, cells were lysed in 10 mM HEPES, pH 7.4, 50 mM NaCl, 1% Triton X-100, 2 mM EDTA, 0.1% β -mercaptoethanol, 1% aprotinin, 50 mM NaF. Glutathione-agarose beads were added to the cell lysates to purify GST-Akt and associated protein(s). GST-Akt and associated protein(s) were eluted in 10 mM glutathione in 50 mM Tris, pH 8.0. Similar experiments were performed with cells transfected with GST-B-Raf and HA-Akt, except that the cells were lysed in 10 mM sodium phosphate, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mg/ml leupeptin, 5 mg/ml aprotinin, 50 mM NaF, 1 mM sodium vanadate. A GST expression vector was included as a negative control. The glutathione-eluted samples were analyzed by Western blot with anti-GST and anti-HA antibodies.

Reporter Assays—In general, HEK293 cells in 35-mm dishes were transfected using a standard calcium phosphate transfection protocol with 36 ng of Gal4-ElkC chimera, 290 ng of a 5xGal4-luciferase reporter, and 145 ng of a B-Raf expression vector or 15 ng of a K-RasV12 expression vector. Total DNA was kept constant by the addition of the appropriate amount of pcDNA3 for all transfections. Luciferase assays were performed using the dual-light luciferase and β -galactosidase reporter gene assay system (Tropix) and were normalized for transfection efficiency using a cotransfected β -galactosidase expression vector (15 ng).

RESULTS

The presence of multiple Akt consensus sites in the amino-terminal regulatory domain of B-Raf led us to investigate whether cross-talk between the Ras signaling pathway and Akt occurs *in vivo*. To determine whether Akt can modulate B-Raf function *in vivo*, we examined the effect of Akt activation and inhibition on B-Raf activity (Fig. 1). Expression of a membrane localized, constitutively active Akt (myrAkt) inhibits B-Raf activity in both actively growing HEK293 cells (Fig. 1A, compare lane 2 with lane 1) and in cells stimulated with epidermal growth factor (Fig. 1A, compare lane 3 with lane 4). The inhibition of B-Raf activity by Akt is dose-dependent (Fig. 1B). A kinase dead version of Akt has no effect on B-Raf activity, suggesting that the catalytic activity of Akt is required to inhibit B-Raf (Fig. 1C). To further test the involvement of endogenous Akt in B-Raf regulation, the PI3K inhibitor LY294002 was used to block the PI3K/Akt pathway. As shown in Fig. 1D, the addition of LY294002 elevates B-Raf activity, suggesting that Akt negatively regulates B-Raf enzymatic activity.

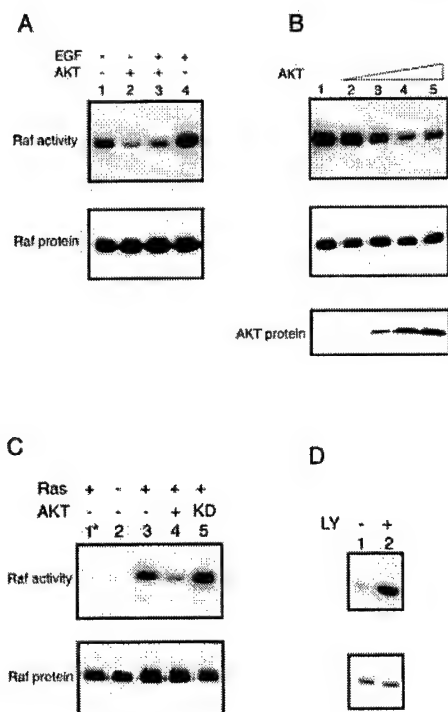


FIG. 1. Akt inhibits B-Raf activity *in vivo*. A, HEK293 cells were transfected with expression vectors for HA-B-Raf and myrAkt, as indicated. B-Raf was immunoprecipitated from lysates and Raf activity assessed by an *in vitro*-coupled kinase reaction in which bacterially expressed GST-Elk-1 was used as a substrate (top panel). Lanes 1 and 2, B-Raf was immunoprecipitated from transfected cells. Lanes 3 and 4, B-Raf was immunoprecipitated from cells after serum starvation and stimulation with epidermal growth factor for 3 min. Western blot of HA-B-Raf proteins used for the *in vitro* kinase is shown in the bottom panel. B, Akt inhibits Raf enzymatic activity in a dose-dependent manner. HEK293 cells were transfected with the expression vector for HA-B-Raf and increasing concentrations of myrAkt, as indicated. B-Raf activity was determined by a coupled *in vitro* kinase reaction (top panel). Western blot of HA-B-Raf proteins used in the *in vitro* kinase reactions (middle panel). Western blot of HA-Akt in cell lysates (bottom panel). C, Akt kinase activity is required to inhibit B-Raf activity. HA-B-Raf was transfected with expression vectors for K-RasV12 or Akt as indicated. Activated Ras stimulates the co-transfected Raf activity (compare lanes 2 and 3). GST-mitogen-activated protein kinase/extracellular signal-regulated kinase kinase was omitted in lane 1 as a control for the specificity of the *in vitro* Raf kinase assays. The Ras-induced Raf activation is inhibited by wild type (lane 4) but not the kinase inactive (lane 5) Akt. D, B-Raf activity is enhanced upon inhibition of PI3K. HA-B-Raf-transfected cells were treated with LY294002 for 1 h. HA-B-Raf was isolated, and kinase activity was determined.

We also examined the association of B-Raf with Akt *in vivo*. As shown in Fig. 2, B-Raf and Akt associate in HEK293 cells overexpressing these proteins. The association between Akt and B-Raf is observed in actively growing HEK293 cells and in HEK293 cells that have been serum-starved or serum-starved and then stimulated with insulin. However, in one orientation, when GST-B-Raf is isolated from HEK293 cells expressing Flag epitope-tagged Akt, the association between B-Raf and Akt is enhanced upon insulin stimulation (data not shown). This may suggest that the association between B-Raf and Akt is sensitive to, but not absolutely dependent on, whether one or both of the proteins is in an activated state.

B-Raf contains three Akt consensus sites, Table I. One site, Ser³⁶⁴ is conserved with c-Raf; however, two sites, Ser⁴²⁸ and Thr⁴³⁹, are unique to B-Raf. To begin to examine the physiological significance of phosphorylation at these sites, the conserved and unique sites were altered to alanine alone and in

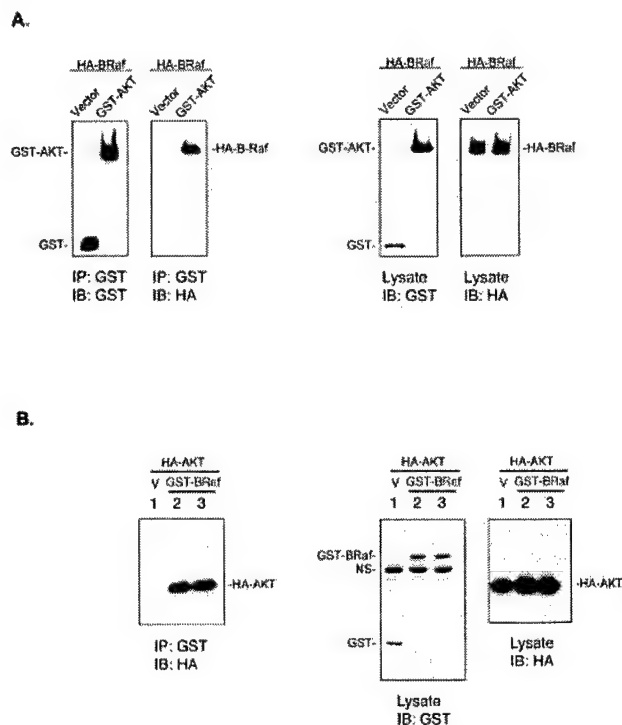


FIG. 2. B-Raf and Akt co-associate *in vivo*. GST-Akt (A) or GST-B-Raf (B) was purified from lysates prepared from HEK293 cells transfected with the indicated constructs using glutathione-Sepharose. The pull-downs were subject to SDS-polyacrylamide gel electrophoresis followed by Western blotting with antibodies directed against the epitope tags on B-Raf and Akt. IP, immunoprecipitate; IB, immunoblot.

TABLE I
Akt consensus sites RXXRXXS/Tφ

Protein	Site	Position
Glycogen-synthase kinase 3β	RPRTTSF	9
BAD	RGRSRSA	136
FKHR	RRRAASM	253
c-Raf	RQRSTST	259
B-Raf	RDRSSSA	364
	REKSSSS	428
	RNRMKTL	439

combination. The effect of the mutations on enzymatic activity *in vitro* and *in vivo* was assessed.

Mutation of Ser³⁶⁴ to Ala (A), of both 428 and 439 to Ala (AA), or of all three Akt consensus sites to Ala (AAA) resulted in a progressive increase in enzymatic activity: B-Raf < B-Raf A < B-Raf AA < B-Raf AAA (Fig. 3). Alteration of Ser³⁶⁴ to Ala (conserved with c-Raf) leads to a modest 2X elevation in enzymatic activity, whereas alteration of both Ser⁴²⁸ and Thr⁴³⁹ to Ala (unique to B-Raf) leads to at least a 19 times elevation of enzymatic activity. Thus, phosphorylation of B-Raf at both the unique and conserved phosphorylation sites is likely to negatively regulate B-Raf enzymatic activity.

Activation of the Ras/Raf/ERK pathway culminates in the phosphorylation of transcription factors, including the ternary complex factor Elk-1 (4). Thus, the degree of activation of the Ras/Raf/ERK pathway can be assessed by examining the transcriptional activity of Elk-1. To address whether the increase in enzymatic activity of the B-Raf alanine mutants *in vitro* correlates with deregulation of enzymatic activity *in vivo*, the effect of the B-Raf mutants on Elk-1-mediated transcriptional activity was assessed by examining the activity of a Gal4-ElkC reporter. Gal4-ElkC contains the carboxyl-terminal domain of

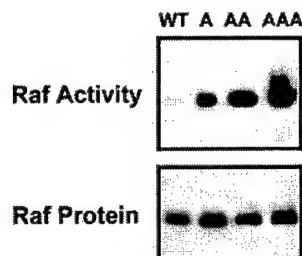


FIG. 3. B-Raf kinase activity is progressively increased upon mutation of the Akt consensus sites. HA epitope-tagged B-Raf, wild type, or mutant at the Akt consensus sites was immunoprecipitated from HEK293 cells, and its enzymatic activity assessed by a coupled *in vitro* kinase reaction in which bacterially expressed GST-Elk-1 was used as substrate. WT, wild type HA-B-Raf; A, HA-B-Raf S364A; AA, HA-B-Raf S428A/T439A; AAA, S364A/S428A/T439A. Raf kinase activity and protein are shown in the *top* and *bottom* panels, respectively.

Elk-1, which includes the transactivation domain of Elk and the ERK phosphorylation sites, fused to the DNA binding domain of the yeast Gal4 protein. The transcriptional activity of Gal4-ElkC is dependent on the phosphorylation of the Elk transactivation domain by ERK (20). As shown in Fig. 4, the B-Raf mutants activate the Elk reporter to a greater extent than wild type B-Raf. Furthermore, B-Raf mutant at all three of the Akt consensus sites activates the Elk-1 reporter to a greater extent than B-Raf mutant at only 364 or B-Raf mutant at both S428A and T439A. The observed increase in reporter activity with the mutants correlates with the progressive increase in enzymatic activity observed in the *in vitro* kinase reactions shown in Fig. 3. Thus, B-Raf proteins altered so as to prevent phosphorylation at the Akt consensus sites activate downstream signaling events.

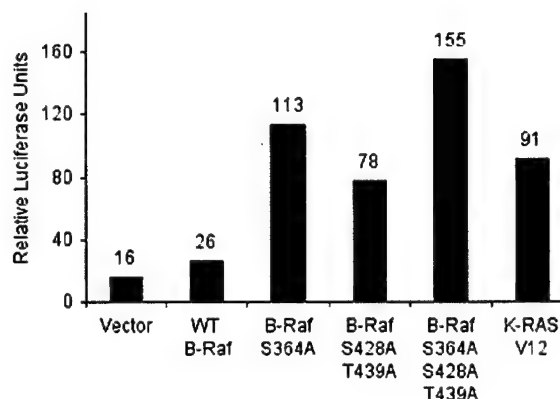
We also examined the effect of the phosphatidylinositol 3-kinase inhibitor LY294002 on activation of the Elk-1 reporter by wild type B-Raf. We consistently observe a modest increase in the ability of B-Raf to activate the Elk-1 reporter in the presence of LY294002 (Fig. 4). This result is consistent with the data in Fig. 1 showing that Akt can negatively regulate B-Raf activity *in vivo*.

In addition, we assessed the deregulation of the enzymatic activity of the B-Raf mutants *in vivo* by assaying ERK activity in the presence and absence of the B-Raf mutants. As expected, ERK activity is elevated in HEK293 cells expressing the B-Raf mutants: B-Raf AAA > B-Raf AA > B-Raf A > B-Raf (Fig. 5).

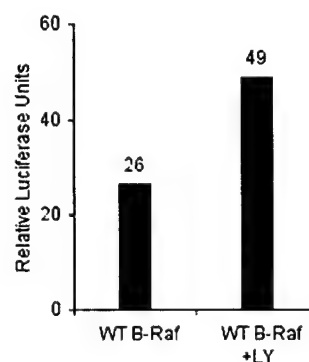
To determine whether B-Raf is a substrate of Akt, *in vitro* kinase reactions were performed. A kinase dead version of B-Raf fused to glutathione *S*-transferase (K/D GST-B-Raf) was used in the *in vitro* kinase reactions. Akt phosphorylates K/D GST-B-Raf but not the control protein GST (Fig. 6). Phosphorylation of K/D GST-B-Raf requires active Akt because phosphorylation was not observed when Akt was prepared from cells in the presence of the phosphatidylinositol 3-kinase inhibitor LY294002.

To determine which of the Akt consensus sites in B-Raf were utilized by Akt, each of the sites was altered to alanine alone and in combination in the context of a K/D GST-B-Raf (see Table I). Each of the mutant proteins was purified from HEK293 cells and used as substrate in *in vitro* kinase reactions with active Akt (harvested from cells after insulin stimulation) or inactive Akt (harvested from cells after treatment with LY294002 and insulin) (Fig. 6A). Quantitation of the *in vitro* kinase reactions by phosphorimage analysis indicates that each of the single mutants B-Raf S364A and B-Raf S428A is phosphorylated to approximately the same extent and that the extent of phosphorylation of each of these mutants is approxi-

A.



B.



C.

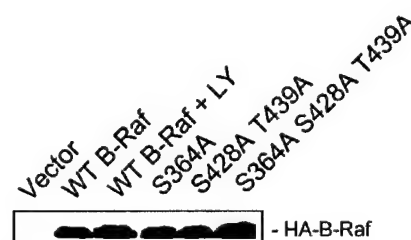


FIG. 4. Activation of Elk-mediated transcription by B-Raf mutants. A, HEK293 cells were transfected with expression vectors for Gal4-ElkC, Gal4-luciferase, and either pcDNA3 (vector control), K-Ras V12 (positive control), wild type (WT)-B-Raf, B-Raf S364A, B-Raf S428A/T439A, or B-Raf S364A/S428A/T439A. B, LY294002 (LY) was added for 24 h prior to harvest, WT B-Raf + LY. Luciferase activity was assayed 36 h after transfection. Luciferase activity was normalized to a co-transfected β -galactosidase expression vector. Shown is the average of two experiments performed in duplicate. C, Western blot of extracts showing expression of HA-B-Raf wild type and mutants.

mately one half that observed for the B-Raf control (Fig. 6B). Taken together, these observations suggest that Akt phosphorylates both Ser³⁶⁴ and Ser⁴²⁸. In contrast, the single mutant B-Raf T439A is not significantly decreased in its phosphorylation compared with the B-Raf control, suggesting that Thr⁴³⁹ is not subject to phosphorylation by Akt. Consistent with the idea that Thr⁴³⁹ is not phosphorylated by Akt, the double B-Raf mutant S428A/T439A is phosphorylated to the same extent as the single B-Raf mutant S428A. Moreover, the extent of phosphorylation of the triple B-Raf mutant S364A/S428A/T439A is approximately one half that observed for each of the single mutants at residues 364 or 428, confirming that Akt phospho-

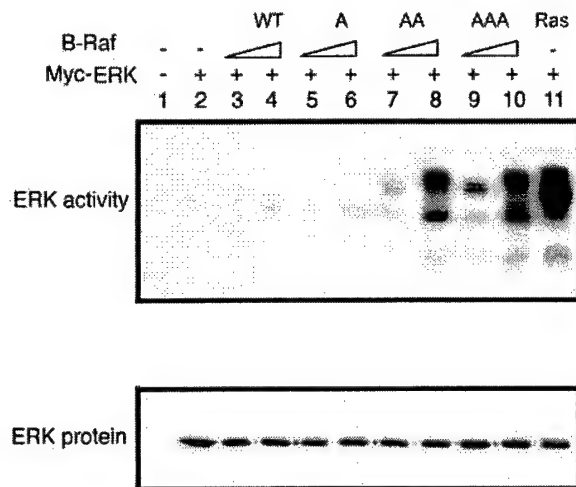


FIG. 5. Mutant Raf has enhanced ability to stimulate ERK activity *in vivo*. HEK293 cells were transfected with expression vectors for wild type or mutant B-Raf proteins or RasV12 and Myc-ERK, as indicated. Myc-ERK was immunoprecipitated, and kinase activity was determined. Western blotting with anti-ERK antibody shows the level of Myc-ERK used in the kinase assay (bottom panel).

rylates both Ser³⁶⁴ and Ser⁴²⁸. Thus, Akt phosphorylates B-Raf *in vitro* at residues Ser³⁶⁴ and Ser⁴²⁸.

DISCUSSION

Here we have demonstrated that *in vivo* and *in vitro* the Ras/Raf signaling pathway is negatively regulated by Akt. First, epidermal growth factor stimulation of B-Raf activity is inhibited by co-expression of Akt. Second, B-Raf enzymatic activity is elevated after treatment of cells with LY294002, a pharmacological inhibitor of PI3K/Akt. Taken together, these observations demonstrate that Akt down-regulates B-Raf activity *in vivo*. Because B-Raf contains three Akt consensus sites located within its amino-terminal regulatory domain, the most likely effect of Akt is a direct phosphorylation at one or more of these sites and a concomitant down-regulation of B-Raf enzymatic activity. We have demonstrated that Akt will phosphorylate two of the three Akt consensus phosphorylation sites *in vitro*, Ser³⁶⁴ and Ser⁴²⁸. Ser³⁶⁴ is conserved with c-Raf, but Ser⁴²⁸ is unique to B-Raf. Substitution of the phosphorylatable residue in the Akt consensus phosphorylation sites with alanine increased B-Raf enzymatic activity, as assessed by *in vitro* coupled kinase assays, activation of the Elk-1 reporter, and activation of ERK activity. Thus, phosphorylation of B-Raf at multiple residues within the amino-terminal regulatory domain negatively regulates its enzymatic activity *in vitro* and *in vivo*.

B-Raf enzymatic activity is further enhanced by combining the alanine substitution mutations within the Akt consensus phosphorylation sites. The seemingly additive nature of the mutations suggests that phosphorylation of these residues is not likely to be ordered but rather phosphorylation at one residue is likely to occur independently of the status of phosphorylation at the other residues. The multiplicity of phosphorylation sites in B-Raf may enable a more flexible regulation (either duration or timing) of B-Raf activity.

Surprisingly, blocking the activation of Akt with the pharmacological inhibitor of phosphatidylinositol 3-kinase, LY294002, did not up-regulate B-Raf activity to the same extent as altering all three Akt consensus sites. The addition of LY294002 only modestly up-regulated B-Raf enzymatic activity (Fig. 1 and data not shown) and Elk-1 reporter activity (Fig. 4), whereas the B-Raf AAA mutant exhibited a striking 20× elevation in enzymatic activity (Fig. 3) and a 6× elevation in

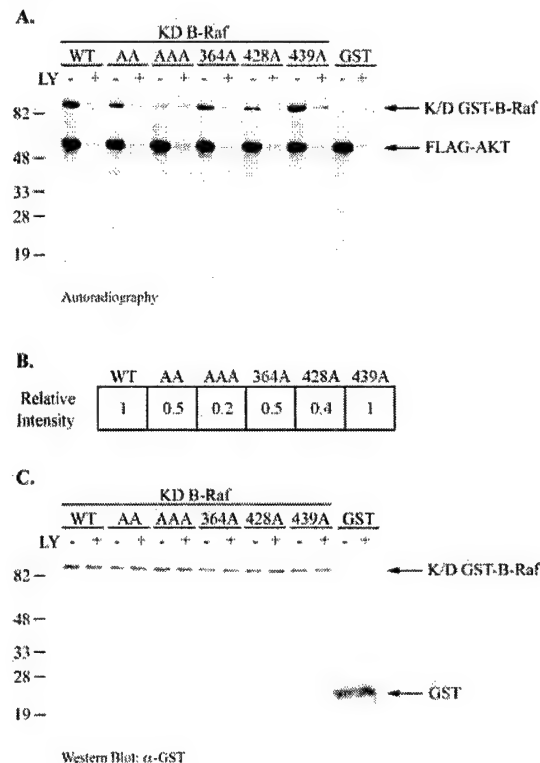


FIG. 6. B-Raf is phosphorylated *in vitro* by Akt at residues 364 and 428. Kinase inactive (K/D) GST-B-Raf fusion proteins were subjected to an *in vitro* kinase reaction with active Akt (–LY) or inactive Akt (+LY). In addition to the K482M mutation to create a kinase-inactive B-Raf, the B-Raf proteins either do not contain additional mutations and are designated WT, for wild type at the Akt consensus sites, or contain mutations at the Akt consensus sites alone (S364A, S428A, or T439A) or in combination (AA, S428A/T439A; AAA, S364A/S428A/T439A). A, autoradiography of the *in vitro* kinase reactions after SDS-polyacrylamide gel electrophoresis and transfer to an Immobilon filter. Arrows show the position of the phosphorylated K/D GST-B-Raf and the autophosphorylated Flag-Akt. B, relative intensity of the phosphorimage signal (–LY lanes) of wild type and mutant K/D GST-B-Raf from the filter in A. The intensity of the signal for each of the mutant proteins was determined relative to K/D GST-B-Raf, which was set to 1.0. C, Western blot analysis of the filter in A, using an anti-GST antibody showing equal loading of the GST-B-Raf substrates.

Elk-1 reporter activity (Fig. 4). Possibly the addition of LY294002 does not block Akt activity in its entirety, a result consistent with observations that Akt can be activated by lipid-independent mechanisms (6). Alternatively, other kinases, in addition to Akt, may be able to regulate B-Raf enzymatic activity by phosphorylation of these sites. Because Thr⁴³⁹ is not phosphorylated by Akt *in vitro*, these other kinases may negatively regulate B-Raf activity at this site.

While our studies were in progress, c-Raf was shown to be phosphorylated by Akt at Ser²⁵⁹ (16), equivalent to Ser³⁶⁴ in B-Raf. The alteration of Ser²⁵⁹ to alanine in c-Raf resulted in a modest 2-fold increase in enzymatic activity. Phosphorylation of Ser²⁵⁹ in c-Raf had been previously shown to decrease the enzymatic activity of c-Raf by promoting its association with 14-3-3 proteins (12). Our results support and extend these observations. First, the regulation of Raf kinases extends to multiple family members; both c-Raf (16) and B-Raf (this report) are subject to phosphorylation and regulation by Akt. Second, of the three Akt consensus sites in B-Raf, only the site conserved with c-Raf (Ser³⁶⁴) lies within a 14-3-3 binding motif (RSXS*XP, where S* represents phosphorylated serine) (12). This suggests that an as yet uncharacterized molecular mechanism, in addition to 14-3-3 binding, is contributing to the

negative regulation of B-Raf at Ser⁴²⁸. Phosphorylation of B-Raf by Akt does not appear to disrupt the association between B-Raf and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase or heat shock protein 90.² Phosphorylation may decrease the association between B-Raf and Ras or Rap or other adaptors and/or signaling molecules, such as Raf kinase inhibitor protein, that regulate Raf activity. Alternatively, phosphorylation may hinder the association of B-Raf with the plasma membrane. Experiments are in progress to test these possibilities. Third, the discordance between the activity of the B-Raf protein mutant at the Akt consensus sites and the activity of B-Raf in the presence of LY294002 might suggest that kinases, in addition to Akt, can act to negatively regulate B-Raf at one or more of these residues.

The Ras signaling pathway regulates cell growth, differentiation, cell survival, cell senescence, and cell death. The sustained *versus* transient activation of the Ras/Raf/ERK pathway is a critically important mediator of signaling specificity and biological outcome (9, 21). We have demonstrated here that Akt can regulate the activity of the kinase cascade downstream of Ras through phosphorylating and inhibiting B-Raf activity. It seems likely that the integration of signals from multiple kinase cascades within a particular cell, such as described here for B-Raf and Akt, will play an important role in modulating the specificity and biological outcome of signal transduction pathways.

² K.-L. Guan, C. Figueroa, and A. B. Vojtek, unpublished observations.

Acknowledgments—We thank Dave Turner and members of the Guan laboratory for helpful comments and suggestions.

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Prenylated Rab Acceptor Protein Is a Receptor for Prenylated Small GTPases*

Received for publication, February 26, 2001, and in revised form, April 27, 2001
Published, JBC Papers in Press, May 2, 2001, DOI 10.1074/jbc.M101763200

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Localization of Ras and Ras-like proteins to the correct subcellular compartment is essential for these proteins to mediate their biological effects. Many members of the Ras superfamily (Ha-Ras, N-Ras, TC21, and RhoA) are prenylated in the cytoplasm and then transit through the endomembrane system on their way to the plasma membrane. The proteins that aid in the trafficking of the small GTPases have not been well characterized. We report here that prenylated Rab acceptor protein (PRA1), which others previously identified as a prenylation-dependent receptor for Rab proteins, also interacts with Ha-Ras, RhoA, TC21, and Rap1a. The interaction of these small GTPases with PRA1 requires their post-translational modification by prenylation. The prenylation-dependent association of PRA1 with multiple GTPases is conserved in evolution; the yeast PRA1 protein associates with both Ha-Ras and RhoA. Earlier studies reported the presence of PRA1 in the Golgi, and we show here that PRA1 co-localizes with Ha-Ras and RhoA in the Golgi compartment. We suggest that PRA1 acts as an escort protein for small GTPases by binding to the hydrophobic isoprenoid moieties of the small GTPases and facilitates their trafficking through the endomembrane system.

Ras proteins (Ha-Ras, K-Ras, N-Ras, TC21, and Ras1/2) regulate cell growth in eukaryotic cells, and perturbation of signaling pathways by mutation and constitutive activation of Ras proteins is a common occurrence in a wide spectrum of human tumors (1). In addition to regulating cell proliferation, Ras proteins also regulate differentiation, cell death, and cell survival (1). The Ras proteins are members of a large superfamily of low molecular weight GTP-binding proteins, which include members that regulate the actin cytoskeleton (Rho and Rac), vesicle trafficking (Rabs), and nuclear transport (Ran).

The biological activity of Ras proteins is controlled by a regulated GTP/GDP cycle (2). The GTP-bound, or active, Ras relays signals to downstream effector proteins. In the case of Ha-Ras, numerous effector proteins have been described, including serine/threonine kinases (c-Raf, A-Raf, and B-Raf), lipid kinases (type I phosphatidylinositol 3-kinase), and guanine nucleotide dissociation stimulators for Ral (Ral GDS¹ and

RGL) (1). Activation of Ras effector pathways leads to proliferation, differentiation, cell death, and cell survival. Which biological outcome predominates is somewhat of a mystery but seems to depend on cell type and the coordinate integration of signaling pathways activated and/or inhibited within a cell.

Members of the Ras superfamily are subject to post-translational modifications. The spectrum of modifications depends on the composition of the carboxyl terminus. In the case of Ha-Ras, the protein is subject to prenylation, proteolysis, carboxyl-methylation, and *S*-acylation/palmitoylation (3). Prenylation is the covalent attachment of farnesyl or geranylgeranyl isoprenoids at or near the carboxyl terminus of the GTPase. For Ras family members, prenylation occurs at a conserved cysteine in the carboxyl-terminal motif termed the CAAX box, where C represents cysteine, A is an aliphatic amino acid, and X is usually Ser, Met, Gln, or Leu (3, 4). Whereas Ha-Ras, N-Ras, K-Ras4B, and yeast Ras2 are farnesylated, most other small GTPases are geranylgeranylated (5). RhoB can be either farnesylated or geranylgeranylated (5). The enzymes that catalyze prenylation, farnesyltransferase and geranylgeranyltransferase-I and II, reside in the cytoplasm.

Prenylation is followed by proteolysis of the carboxyl-terminal tripeptide and methylation of the newly generated carboxyl-terminal amino acid in the endoplasmic reticulum (ER) (3, 4). Some Ras proteins, including Ha-Ras, N-Ras, yeast Ras2, and Rac1 are further modified by *S*-acylation (palmitoylation) of cysteine(s) located near the carboxyl terminus of the protein (3, 4). *S*-acylation is likely to occur in the ER, since Erf2, a novel integral membrane protein required for palmitoylation of Ras in yeast, is localized to the ER (6). Other proteins, such as K-Ras4B, that are not subject to *S*-acylation have a polybasic stretch of amino acids, also located near the CAAX box, within the 20-amino acid hypervariable domain of the Ras proteins. The combination of two signals, prenylation and *S*-acylation or prenylation and a polybasic stretch of amino acids, are required for plasma membrane targeting of Ras proteins. Ras proteins (Ha-Ras, N-Ras) transit through the ER and Golgi and finally reach the plasma membrane via exocytic transport vesicles (7, 8). The trafficking of K-Ras4B, however, is unlikely to involve the Golgi compartment; rather, once prenylated and further modified in the ER, the trafficking of K-Ras4B to the plasma membrane involves a microtubule network (9, 10).

As a consequence of the post-translational modifications, Ras is membrane-localized. In mammalian cells, membrane localization of Ras is necessary for transformation and differentiation. Membrane localization of Ras creates a docking site for effectors, including the Raf kinases and phosphatidylinositol

* This work was supported by a grant from the Walther Cancer Research Institute and U.S. Army Medical Research and Materiel Command Grant DAMD17-98-1-8319. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: GDS, guanine nucleotide dissociation

stimulator; ER, endoplasmic reticulum; GDI1, guanine nucleotide dissociation inhibitor 1; GST, glutathione *S*-transferase; PRA1, prenylated Rab acceptor protein; SOS, son of sevenless; GFP, green fluorescent protein; eGFP, enhanced GFP; PCR, polymerase chain reaction; IP, immunoprecipitation.

With this report, we demonstrate that PRA1 is not a specific partner for Rab proteins but also partners with other members of the Ras superfamily. The association of Ras family members with PRA1 requires their post-translational modification by prenylation. Further, our results suggest that an isoprenoid moiety, either farnesyl or geranylgeranyl, is the critical recognition target for PRA1. The interaction between Ha-Ras and PRA1 and between RhoA and PRA1 is observed *in vivo* by co-precipitation experiments and co-localization in mammalian cells. The co-localization of Ha-Ras and PRA1 and RhoA and PRA1 to the Golgi compartment suggests that PRA1 may play a role in facilitating the trafficking of small GTPases through the endomembrane system. In addition, we have cloned the yeast PRA1 gene and demonstrate that, like its higher eukaryotic counterpart, the yeast PRA1 protein also interacts with multiple small GTPases, and the interaction requires prenylation of the small GTPase. Thus, PRA1 is predicted to play a conserved role in the biology of small GTPases in all eukaryotic cells.

Microscopy—COS cells were transiently transfected with expression vectors for eGFP-PRA1 and either GST-Ha-Ras or GST-RhoA, pCS2 + eGFP-PRA1, pEBG-3X-Ha-RasV12, and pEBG-RhoA163, respectively. Cells were fixed 36 h post-transfection on polylysine-coated coverslips with 3.7% paraformaldehyde (Sigma) in phosphate-buffered saline, permeabilized for 15 min with 0.1% Triton X-100 in phosphate-buffered saline, and blocked with 10% goat serum in phosphate-buffered saline for 30 min. Fixed and permeabilized cells were incubated with mono-

clonal hybridoma supernatant harvested from 9E10 cells (Myc epitope tag antibody) or with anti-GST antisera, washed in phosphate-buffered saline, and then incubated with Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.). After washing, the coverslips were mounted on glass slides with Prolong (Molecular Probes, Inc., Eugene, OR). The cells were viewed on a Noran OZ laser-scanning confocal microscope, and the data were collected in a UNIX-based Silicon Graphics INDY R5000 work station or viewed on a Nikon deconvolution microscope. The figures were prepared using Photoshop 5.5 (Adobe), and the relative intensities have been adjusted to visualize spatial overlap.

RESULTS

Rip69 was identified as a Ha-Ras-interacting protein in a yeast two-hybrid screen of a mouse embryo cDNA library (14). Among the clones recovered in this screen were those that required an intact Ras effector domain for the Ras-Rip interaction. The proteins encoded by these isolates, which include Raf family members (14), the catalytic subunit of phosphatidylinositol 3-kinase (21), and guanine nucleotide exchange factors for Ral, were subsequently shown to be direct downstream targets of Ras. In addition, isolates were recovered that associated with Ras proteins mutated in the effector domain; among these was Rip69. The lack of a requirement for an intact Ras effector domain for the Ras-Rip69 protein-protein interaction suggests that the Rip69 isolate does not encode an effector of Ras function but is more likely to be either a regulator of Ras or a protein involved in the trafficking of Ras to the plasma membrane. Rip69 encodes residues 20–186 of prenylated Rab acceptor protein PRA1.

Three published reports demonstrate that PRA1 interacts with members of the Rab family of GTPases, including Rab1, Rab3A, Rab5, and Rab6 (15, 17, 22). Among small GTPases, PRA1 is thought to be a relatively specific partner for Rab proteins because, in two reports, PRA1 failed to interact with other members of the Ras superfamily, Ras, Rho, and Rac (15, 17).

Because we had identified PRA1 in a two-hybrid screen with Ha-Ras, we investigated the ability of the full-length mouse PRA protein, PRA1, to interact with RhoA and TC21. In addition to interacting with Ha-Ras, PRA1 interacts with TC21 and RhoA, Fig. 1. These results indicate that the interaction of PRA1 with small GTPases is not specific to Rab proteins; instead, PRA1 interacts with multiple members of the Ras superfamily, including Ha-Ras, RhoA, and TC21. Although Rap1a does not interact with PRA1 in a yeast two-hybrid experiment, Rap1a does interact with PRA1 *in vivo* in mammalian cells (as described below and in Fig. 2). Perhaps the LexA-Rap1a fusion protein is not prenylated efficiently in yeast, or it may not localize to the nucleus as efficiently as the unprenylated form.

To confirm our yeast two-hybrid experiments, we examined the association of Ha-Ras and RhoA with PRA1 *in vivo*. As shown in Fig. 2, A and B, Ha-Ras and PRA1 and RhoA and PRA1 associate in HEK293 cells overexpressing these proteins. In addition, because we did not observe an association of PRA1 with Rap1a in yeast, we examined the interaction in mammalian cells. As shown in Fig. 2C, Rap1a and PRA1 co-associate *in vivo* in HEK293 cells. Thus, PRA1 associates with multiple members of the Ras superfamily *in vivo*.

Ha-Ras proteins undergo a series of post-translational modifications: 1) attachment of an isoprenoid farnesyl moiety to a cysteine located 4 residues from the carboxyl terminus of the protein; 2) removal of the carboxyl-terminal tripeptide; 3) methylation of the carboxyl group of the farnesylated cysteine; and, 4) palmitoylation of two cysteines near the carboxyl terminus of the protein, Cys¹⁸¹ and Cys¹⁸⁴ (3, 4). To investigate the requirement for post-translational modification of Ha-Ras on the association of PRA1 with Ha-Ras, we examined the

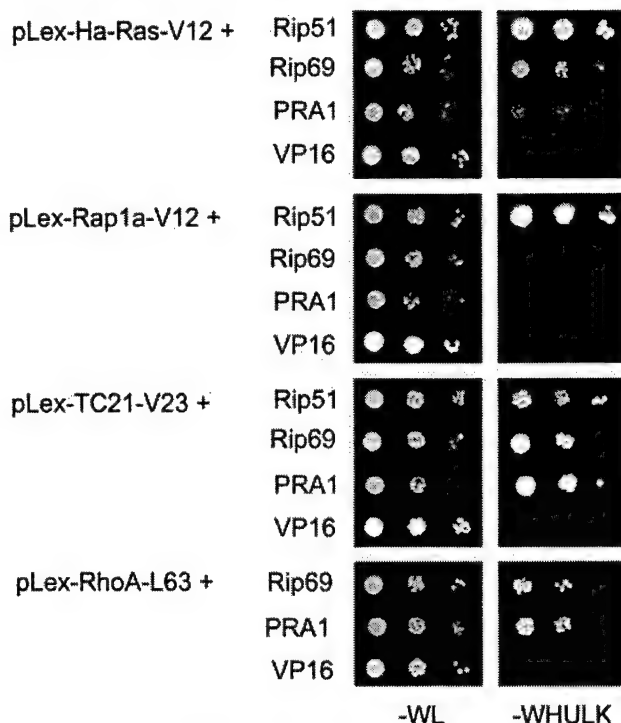


FIG. 1. PRA1 interacts with multiple members of the Ras superfamily of small GTPases. The interaction of small GTPases with PRA1 was assessed by yeast two-hybrid analysis. The *S. cerevisiae* reporter strain L40 was transformed with a plasmid that expresses a Ras family member fused to the LexA DNA binding domain (pLex-RasV12, pLex-Rap1aV12, pLexTC21V23, or pLexRhoAL63) and plasmids that express the VP16 activation domain alone (pVP16), the VP16 activation domain fused to the amino-terminal domain of c-Raf (Rip51, positive control), the domain of PRA1 recovered in the yeast two-hybrid screen (Rip69), or full-length mouse PRA1 (PRA1). Yeast transformants were grown overnight at 30 °C in liquid culture maintaining selection for the plasmids (YC-WL), and dilutions of the overnight cultures were plated to YC-WL plates or to YC-WHULK plates to assess the activation of the *HIS3* reporter. A positive association between the LexA fusion protein and the VP16 fusion protein leads to growth in the absence of histidine (YC-WHULK plates). Activated Ras, TC21, and RhoA associate with PRA1 and Rip69.

association of PRA1 with mutant Ras proteins missing key post-translational modifications (Fig. 3). Ha-RasV12ΔCAAX is an activated Ras that lacks all post-translational modifications. Ha-RasV12K6 is an activated Ras with polylysine substituted for Ser¹⁸⁹, the last residue of the CAAX box; this mutant Ras is palmitoylated but not farnesylated (23). Ha-RasV12 S181 S184 is an activated Ras that is farnesylated, proteolyzed, and methylated but not palmitoylated (23). PRA1 interacts with Ha-RasV12 S181 S184 but not Ha-RasV12ΔCAAX or Ha-RasV12K6 (Fig. 3). Thus, Ras proteins that are not palmitoylated are still capable of associating with PRA1, indicating that palmitoylation of Ras is not required for the association of the proteins. Ras proteins that are not farnesylated or are not modified in any way do not interact with PRA1, suggesting that farnesylated Ras is the binding partner for PRA1.

In order to determine whether the modified carboxyl-terminal tetrapeptide of Ha-Ras is both necessary and sufficient for recognition by PRA1, we assessed the association of PRA1 with GFP-CAAX, a GFP protein to which only the last 4 amino acids of Ha-Ras, CVLS, were added. This tetrapeptide is both necessary and sufficient for farnesyltransferase and geranylgeranyl transferase I to recognize and prenylate Protein A and G α , which have been mutated to end in a CAAX sequence (24, 25). Likewise, we expect that in yeast this tetrapeptide will be

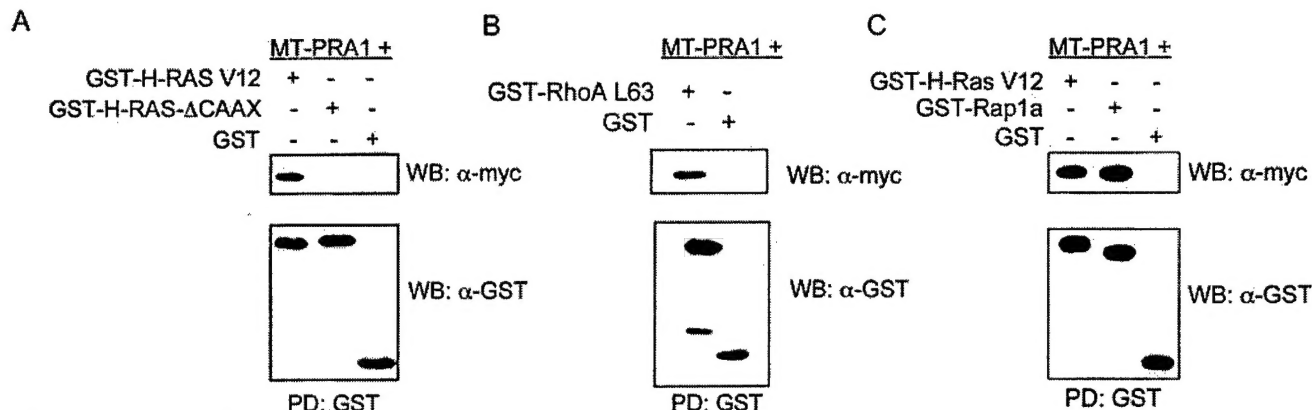


FIG. 2. **Ha-Ras, RhoA, and Rap1a interact with PRA1 *in vivo*.** GST or GST fusion proteins were purified from lysates prepared from HEK293 cells transfected with the indicated constructs using glutathione-Sepharose. The pull-downs were subject to SDS-PAGE followed by Western blotting with antibodies directed against the epitope tags on Ha-Ras, RhoA, or Rap1a (GST) or PRA1 (Myc). A, GST-Ha-RasV12, but not GST or GST-Ha-RasV12CAAX, associates with PRA1. B, GST-RhoA L63 but not GST associates with PRA1. C, GST-Rap1a but not GST associates with PRA1.

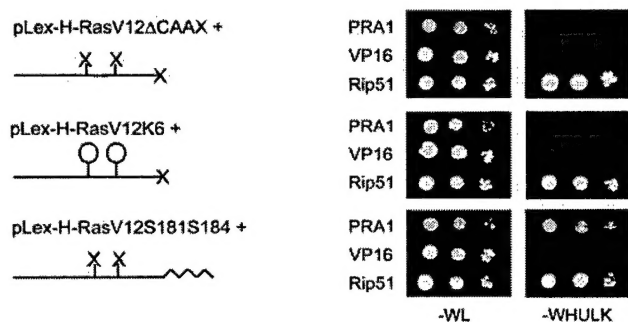


FIG. 3. **Farnesylation of Ha-Ras but not palmitoylation is required for the Ha-Ras/PRA1 association.** The yeast L40 strain was transformed with the various plasmid combinations, and the activation of the *HIS3* reporter was assessed. pLex-Ha-RasV12CAAX is a fusion of the LexA DNA binding domain to an activated Ras, which lacks the CAAX box; this mutant Ras is not post-translationally modified. pLex-Ha-RasV12K6 is a fusion of LexA to an activated Ras with polylysine substituted for Ser¹⁸⁹, the last residue of the CAAX box; this mutant Ras is palmitoylated but not farnesylated. pLex-Ha-RasV12S181S184 is a fusion of LexA to an activated Ras that is farnesylated, proteolyzed, and methylated but not palmitoylated. VP16-PRA1 is a fusion of the VP16 activation domain to full-length PRA1. Rip51 expresses the amino-terminal domain of c-Raf fused to VP16. PRA1 interacts with Ha-RasV12S181S184 but not Ha-RasV12CAAX or Ha-RasV12K6. LexA-Ha-RasV12CAAX and LexA-Ha-RasV12K6 are able to interact with Rip51; thus, lack of interaction of these mutant proteins with PRA1 is not simply due to lack of expression or inability to localize to the correct cellular compartment.

recognized and prenylated by yeast farnesyltransferase and probably proteolyzed and methylated because mammalian Ha-Ras when expressed in yeast is appropriately modified. As shown in Fig. 4, PRA1 interacts with GFP-CAAX but not GFP. This result suggests that the molecular determinants for the binding of PRA1 reside within the CAAX box of Ha-Ras and that a modified tetrapeptide (farnesylated, proteolyzed, and/or methylated) is both necessary and sufficient to mediate the protein-protein interaction.

The sole post-translational modification in common among the PRA1 interacting proteins, Rabs, Ras, Rho, and TC21, is isoprenylation, the attachment of farnesyl or geranylgeranyl moieties to the proteins (Table I). To demonstrate that post-translational processing events subsequent to prenylation (proteolysis and/or methylation) are not essential for PRA1 binding, we examined the association of PRA1 with GFP-CVYS. GFP-CVYS contains a substitution of Tyr for Leu at the A₂ position of the CA₁A₂X box. A K-Ras Tyr¹⁸⁷ CAAX box mutant

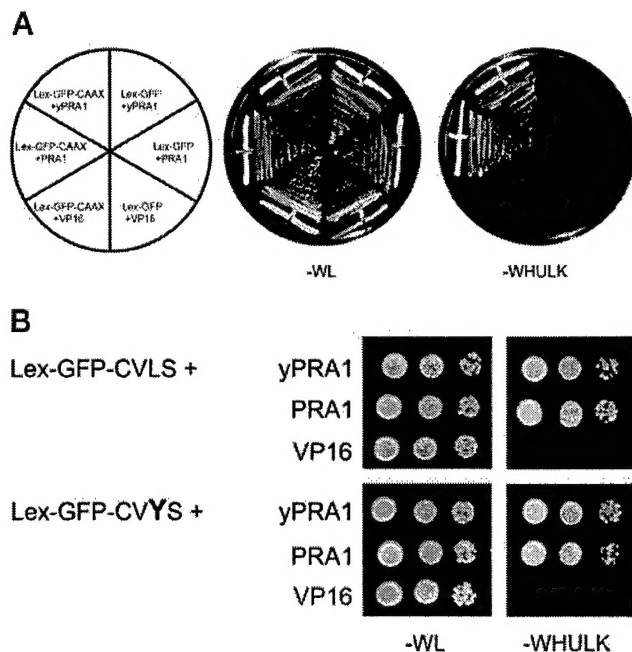


FIG. 4. **A, the addition of the Ha-Ras CAAX box to GFP is necessary and sufficient to mediate the association of PRA1 with GFP. B, post-translational events subsequent to prenylation are not essential for PRA1 binding.** The yeast L40 strain was transformed with the various plasmid combinations, and the activation of the *HIS3* reporter was assessed. pLexA-GFP CAAX expresses the LexA DNA binding domain fused to a GFP to which the carboxyl-terminal Ha-Ras tetrapeptide CVLS has been added. pLexA-GFP expresses the LexA DNA binding domain fused to GFP. pLexA-GFP-CVYS expresses the LexA DNA binding domain fused to GFP to which a mutant Ha-Ras CAAX box, CVYS, has been added. LexA-GFP-CVYS is prenylated but not further processed (methylated and/or proteolyzed). pVP16-yPRA1 expresses the VP16 activation domain fused to full-length yeast PRA1. pVP16-PRA1 expresses the VP16 activation domain fused to full-length mouse PRA1. pVP16, vector control. Both yeast and mouse PRA1 associate with GFP CAAX and with GFP-CVYS but not GFP.

protein is prenylated but not further processed (33). As shown in Fig. 4B, PRA1 interacts with GFP-CVYS. Taken together, our observations suggest that an isoprenoid moiety, either farnesyl or geranylgeranyl, is the critical recognition target for PRA1.

To confirm the prenylation dependence observed in yeast two-hybrid experiments, modified and unmodified GST-Ha-Ras was purified from mammalian cells after Triton X-114

TABLE I
PRA1 associates with multiple GTPases: The sole feature in common is prenylation

The carboxyl-terminal sequence of each GTPase is shown, and the post-translational modifications each GTPase undergoes are indicated. F, farnesylation; G, geranylgeranylation; AAX, proteolysis following prenylation; Y, yes; N, no. Two-hybrid interaction, PRA1 + small GTPase. The two-hybrid interaction data for Ha-Ras, TC21, RhoA, and Rap1a are derived from Fig. 1 of this report and from published reports (15–17) for Rab3A and Rab1 (*).

GTPase	C-terminal sequence	Prenylation	AAX	Methylation	Palmitoylation	Two-hybrid interaction
Ha-Ras	DESGPGCMSCKCVLS	F	Y	Y	Y	++
TC21	TRKEKDKKGCHCVIF	F	Y	Y	Y	++
RhoA	LQARRGKKKSGCLVL	G	Y	Y	N	++
Rap1a	PVEKKKPKKSCLLL	G	Y	Y	N	— ^a
Rab3a	QLSDQQVPPHQDCAC	G	N	Y	N	+*
Rab1	KIDSTPVKSASGGCC	G	N	N	N	+*

^a Although Rap1a fails to interact with PRA1 in a yeast two-hybrid test, Rap1a interacts with PRA1 *in vivo* in mammalian cells (see Fig. 2 and "Results").

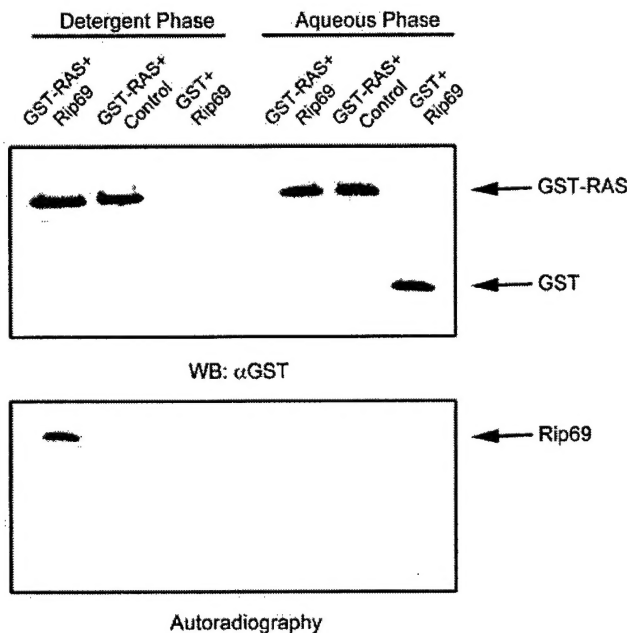


FIG. 5. Post-translationally processed Ha-Ras associates with PRA1. GST-Ha-Ras or GST was transiently expressed in HEK293 cells, and processed and unprocessed proteins were prepared by a Triton X-114 partitioning assay. Hydrophobic proteins partition into the detergent phase, whereas hydrophilic proteins partition into the aqueous phase of Triton X-114. The processed Ha-Ras partitions into the detergent phase, whereas the unprocessed Ha-Ras is found in the aqueous phase. GST, a soluble protein, partitions exclusively into the aqueous phase. The GST fusion protein from equal proportions of each phase was captured on glutathione-Sepharose resin and mixed with VP16-Rip69 (amino acids 20–186 of PRA1 fused to VP16) or VP16 (control), prepared in reticulocyte lysates in the presence of [³⁵S]methionine. The resin was washed after incubation to remove unbound proteins, and then the proteins in each of the binding reactions were resolved by SDS-PAGE and subject to autoradiography (lower panel) followed by immunoblotting with anti-GST antiserum and detected by enhanced chemiluminescence (upper panel). GST-Ha-Ras purified from the Triton X-114 detergent, but not aqueous, phase interacts with VP16-Rip69. GST does not associate with Rip69.

partitioning. GST-Ha-Ras from the detergent phase (modified Ras) interacted with PRA1 *in vitro*, whereas GST-Ha-Ras from the aqueous phase (unmodified Ras) did not interact with PRA1 *in vitro* (Fig. 5). Taken together, our observations in yeast and *in vitro* indicate that PRA1 preferentially associates with post-translationally modified (prenylated) Ras.

Sequences homologous to PRA1 are present in *S. cerevisiae*, *Schizosaccharomyces pombe*, *Arabidopsis thaliana*, *Drosophila melanogaster*, and *Caenorhabditis elegans*. To determine if the association of PRA1 with small GTPases was conserved, we

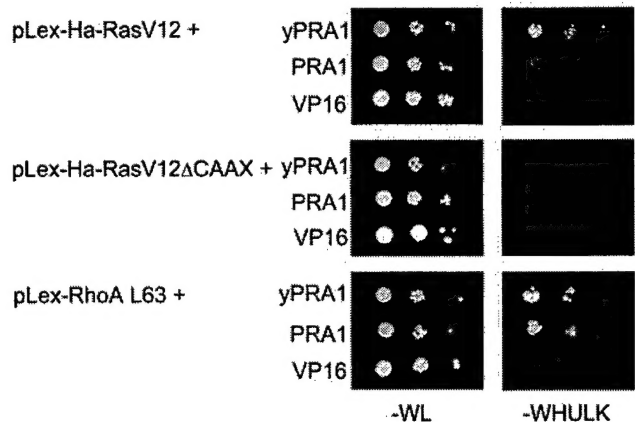


FIG. 6. The association of PRA1 with Ha-Ras and RhoA is conserved in evolution. The yeast L40 strain was transformed with the various plasmid combinations, and the activation of the *HIS3* reporter was assessed. The yeast homolog of PRA1, yPRA1, associates with both Ha-Ras and RhoA. The association of Ha-Ras with yPRA1 is observed only with the modified (Ha-RasV12) and not the unmodified (Ha-RasV12ΔCAAX) Ras.

cloned the yeast homolog and assessed its ability to interact with Ha-Ras and RhoA. The yeast PRA1 protein, yPRA1, interacts with both Ha-Ras and RhoA (Fig. 6). The association of Ha-Ras and yPRA1 is dependent on the post-translational modification of Ras because Ha-RasΔCAAX is unable to bind to yPRA1 (Fig. 6). Thus, the interaction of PRA1 with Ras family members is conserved in evolution.

Recent studies have demonstrated an association of Ha- and N-Ras with endomembranes (8, 26). Ha- and N-Ras are prenylated in the cytoplasm and then undergo additional modifications as they transit through the ER and then Golgi. RhoA also is present in the ER and/or endosomal vesicles (27, 28). The prenylation-dependent association of small GTPases with PRA1, together with the ability of PRA1 to interact with small GTPases with effector domain mutations (a result that strongly suggests that PRA1 is not an effector of the small GTPases with which it interacts) suggested to us that PRA1 may function in an endomembrane compartment. Therefore, we compared the spatial distribution of PRA1 and Ha-Ras and RhoA in COS1 cells. Both Ha-Ras and PRA1 exhibit perinuclear staining, indicative of Golgi localization, and Ha-Ras and PRA1 co-localize in this perinuclear region, Fig. 7A. In addition, RhoA and PRA1 also co-localize in the Golgi compartment (Fig. 7B). Recent studies have shown that PRA1 is present in the Golgi compartment (16), and our studies using Golgi markers confirm this (data not shown).

The 15 carbon farnesyl and 20 carbon geranylgeranyl isoprenoid groups are hydrophobic, although the hydrophobicity of

A



B



FIG. 7. Subcellular localization of Ha-Ras V12, RhoA L63 and PRA1. COS1 cells were transiently transfected with expression vectors for eGFP-PRA1 and either GST-RasV12 (A) or GST-RhoA L63 (B). GST-Ha-RasV12 and GST-RhoA L63 were detected by indirect immunofluorescence using a rabbit anti-GST primary antibody and CY3 secondary antibody. eGFP1-PRA1 was detected by epifluorescence. A similar localization of Ha-RasV12, RhoA L63, and PRA1 to that shown here in fixed cells was observed when eGFP fusions to Ha-RasV12, RhoA L63, and PRA1 were visualized in live cells (data not shown).

these groups do not support stable membrane localization. Since PRA1 specifically recognizes the isoprenoid moieties of small GTPases, the binding of PRA1 to prenylated small GTPases may facilitate their trafficking through the endomembrane system by masking the hydrophobicity of the isoprenoid groups.

DISCUSSION

With this report, we demonstrate that PRA1 associates with multiple members of the Ras superfamily. We show that PRA1 associates with Ha-Ras, RhoA, TC21, and Rap1a. Others have shown that PRA1 associates with Rab family members (15–17). The sole feature in common among these small GTPases is the presence of farnesyl or geranylgeranyl isoprenoid moieties covalently linked to cysteines located at or near the carboxyl terminus of the small GTPases (Table I). Our Ras CAAX box mutant studies (Fig. 3), Triton-X114 partitioning studies (Fig. 5), and GFP-CAAX/PRA1 protein-protein interaction studies (Fig. 4) are consistent with the farnesyl or geranylgeranyl moiety of the small GTPase as being the key recognition target for PRA1.

Small GTPases are not the only proteins subject to modification by prenylation. Prelamin A, lamin B, γ subunits of heterotrimeric G proteins, and serine/threonine kinases such as LKB1 are prenylated (3, 29). It will be interesting to determine whether PRA1 associates with proteins outside the Ras superfamily.

A search of the data bases reveals the presence of PRA1 homologs in many organisms. PRA1 homologs are present in both budding and fission yeast, in *A. thaliana*, *C. elegans*, *D. melanogaster*, and *Mus musculus*. yPRA1, the PRA1 homolog from budding yeast, interacts with multiple prenylated small GTPases (Fig. 6). Thus, the interaction of PRA1 with multiple small GTPases is conserved in evolution, and this conservation of the association suggests that the function of the PRA1-small GTPase interaction may also be conserved in evolution. Experiments are in progress to genetically decipher the physiological role of PRA1 in yeast.

The association of PRA1 with multiple small GTPases *in vitro* and *in vivo* suggests that PRA1 has a role that is held in common with all small GTPases that it interacts with. The feature in common among the small GTPases that PRA1 interacts with is their post-translational modification by farnesyl or geranylgeranyl isoprenoid moieties. We suggest that PRA1 binds prenylated GTPases to act as an escort protein for the GTPases. Biochemical fractionation and localization experi-

ments show that PRA1 is found both in the cytoplasm and in the Golgi (16). PRA1 may associate with small GTPases after prenylation occurs in the cytoplasm to "solubilize" the hydrophobic prenyl motifs and thus facilitate trafficking through the endomembrane system. Alternatively, PRA1 may function in the Golgi to facilitate the entry of small GTPases into vesicles for transport to cellular compartments. PRA1 is found in synaptic vesicle membranes, so PRA1 may cycle with the Ras proteins in the exocytic vesicles out to the plasma membrane (30).

PRA1 also associates with GDI1 (16). The addition of recombinant GDI1 to membranes prepared from PC12 cells was effective at removing Rab3A from the membrane; the addition of PRA1 blocked the ability of GDI to extract Rab3A (16). These observations suggest that the opposing actions of GDI1 and PRA1 may influence the membrane localization of the Rab proteins. GDIs have been described that solubilize membrane-associated Rho proteins (31). Possibly, the opposing action of GDI and PRA1 is a general mechanism for regulating the membrane status of small GTPases that are acted upon by GDIs.

The prevalence of Ras mutations in human tumors and the requirement of plasma membrane localization of Ras for cellular function suggested early on that inhibitors of farnesyltransferase might be a promising class of cancer therapeutics (5). Some of these inhibitors of farnesyltransferase are presently being evaluated in phase II clinical trials. However, small GTPases, such as RhoC, which has been recently associated with promotion of metastasis (32), are geranylgeranylated, so inhibitors of geranylgeranyl transferase may also prove to be a promising class of cancer therapeutics. In addition, some GTPases are "switch-hitters," subject to either farnesylation or geranylgeranylation. Therefore, inhibiting one pathway may not necessarily prove efficacious in cancer treatment. PRA1 binds both farnesylated and geranylgeranylated small GTPases and may act as a receptor or escort protein for Ras superfamily members. Thus, inhibitors directed against PRA1 may provide a means to pharmacologically intervene to inhibit the signaling pathways activated aberrantly by both farnesylated and geranylgeranylated small GTPases, which can result in sustained cell growth (tumorigenesis) and metastasis.

Acknowledgments—We thank Bob Fuller and David Turner for helpful comments and suggestions. We thank Jonathan A. Cooper for support during the initial cloning of Rip69/PRA1.

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